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Molecular Distinctions between Stasis and Telomere Attrition Senescence Barriers Shown by Long-term Culture of Normal Human Mammary Epithelial Cells

James C. Garbe,¹ Sanchita Bhattacharya,¹ Batul Merchant,¹ Ekaterina Bassett,¹ Karen Swisshelm,² Heidi S. Feiler,¹ Andrew J. Wyrobek,¹ and Martha R. Stampfer¹

¹Life Sciences Divisions, Lawrence Berkeley National Laboratory, Berkeley, California and ²Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado

Abstract

Normal human epithelial cells in culture have generally shown a limited proliferative potential of ~10 to 40 population doublings before encountering a stress-associated senescence barrier (stasis) associated with elevated levels of cyclin-dependent kinase inhibitors p16 and/or p21. We now show that simple changes in medium composition can expand the proliferative potential of human mammary epithelial cells (HMEC) initiated as primary cultures to 50 to 60 population doublings followed by p16-positive, senescence-associated β -galactosidase-positive stasis. We compared the properties of growing and senescent pre-stasis HMEC with growing and senescent post-selection HMEC, that is, cells grown in a serum-free medium that overcame stasis via silencing of p16 expression and that display senescence associated with telomere dysfunction. Cultured pre-stasis populations contained cells expressing markers associated with luminal and myoepithelial HMEC lineages *in vivo* in contrast to the basal-like phenotype of the post-selection HMEC. Gene transcript and protein expression, DNA damage-associated markers, mean telomere restriction fragment length, and genomic stability differed significantly between HMEC populations at the stasis versus telomere dysfunction senescence barriers. Senescent isogenic fibroblasts showed greater similarity to HMEC at stasis than at telomere dysfunction, although their gene transcript profile was distinct from HMEC at both senescence barriers. These studies support our model of the senescence barriers encountered by cultured HMEC in which the first barrier, stasis, is retinoblastoma-mediated and independent of telomere length, whereas a second barrier (agonescence or crisis) results from telomere attrition leading to telomere dysfunction. Additionally, the ability to maintain long-term growth of genomically stable multilineage pre-stasis HMEC populations can greatly enhance experimentation with normal HMEC. [Cancer Res 2009;69(19):7557–68]

Introduction

Human epithelial cell culture systems provide experimentally tractable means to examine the processes involved in normal human cell biology, aging, and carcinogenesis, but the limited

proliferative potential of epithelial cells cultured from normal human tissues has constrained such studies. Whereas cultured human fibroblastic cells generally display 50 to 80 population doublings, most published reports on cultured human epithelial cells have shown active growth for only 10 to 30 population doublings (1–3), although keratinocytes have proliferated for up to 45 population doublings (4).

Our previous work culturing human mammary epithelial cells (HMEC) derived from reduction mammoplasty tissues has shown active growth for 10 to 25 population doublings, depending on culture condition, before an initial proliferative arrest (1, 2, 5). Under specific conditions, for example, exposure to the chemical carcinogen benzo(a)pyrene (6, 7), or growth in the serum-free medium MCDB170 (2), rare cells have emerged that lack expression of the cyclin-dependent kinase inhibitor p16^{INK4A} and are capable of proliferation for an additional 30 to 70 population doublings before encountering a second proliferative arrest (5, 8, 9). The spontaneous emergence from MCDB170-grown HMEC of rare cells with silenced p16 expression was originally called selection and the ensuing proliferative population post-selection (2). Due to their increased population doubling potential, post-selection p16-negative HMEC have been commonly used for examination of supposedly normal finite lifespan HMEC; however, post-selection HMEC can differ significantly from normal HMEC in culture and *in vivo* (9–14), prompting the need to develop improved methods for normal HMEC culture.

Based on molecular analysis of HMEC phenotypes at these two proliferative barriers, we proposed a new model of the senescence barriers encountered by cultured HMEC (9). In this model, the first barrier, which we have called stasis, is stress-associated and mediated by retinoblastoma. The population is arrested in G₁, has a low labeling index, and does not show genomic instability or critically short telomeres; virtually all the nonproliferative cells express p16 protein (5). The second barrier is associated with critically shortened telomeres, evidence of DNA damage and genomic instability, and is a consequence of telomere dysfunction due to ongoing telomere attrition (5, 9, 15, 16). In the presence of functional p53, this barrier has been called agonescence (17); cell populations remain viable, with a labeling index of ~15% and arrest at all phases of the cell cycle. If p53 function is abrogated, cells enter crisis and eventually die (9). HMEC at both stasis and agonescence express senescence-associated β -galactosidase (SA- β -Gal) activity and display a senescent morphology (9); thus, these markers do not distinguish between these distinct senescence barriers.

Based on this model of the senescence barriers, we hypothesized that the limited and variable population doubling potential of

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Current address for E. Bassett: Geron Corp., Menlo Park, California.

Requests for reprints: Martha R. Stampfer, Life Sciences Divisions, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720-8174. Phone: 510-486-7273; Fax: 510-486-4545; E-mail: mrstampfer@lbl.gov.

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pre-stasis HMEC was due to variable stress exposure under different culture conditions; we therefore looked to determine culture conditions that could increase the population doubling potential of pre-stasis HMEC. We now report that simple changes in the culture conditions can permit active growth of pre-stasis HMEC for up to 60 population doublings before a p16-positive stasis arrest (low labeling index, G₁ arrest, normal karyotype, and variable telomere length). Cells with markers of both myoepithelial and luminal epithelial lineages are present in these populations. This enhanced proliferative potential permits generation of large standardized early-passage cell batches that may facilitate experimental examination of normal cultured HMEC and how these cells transform to malignancy.

Pre-stasis HMEC were further analyzed for gene expression profiling and evidence of DNA damage at increasing passage levels, and these data compared with post-selection HMEC and isogenic human mammary fibroblast cells (HMFC). Our results show significant differences between pre-stasis and post-stasis HMEC populations. Cells at agonescence had greater evidence of DNA damage than cells at stasis, and distinct gene expression patterns. Gene expression in HMFC at senescence was distinct from HMEC at both stasis and agonescence, although the senescent HMFC resembled HMEC at stasis rather than agonescence in other properties. These data support our model of the mechanistically distinct senescence barriers encountered by cultured HMEC, one associated with stresses and one associated with telomere dysfunction due to telomere attrition. Altogether, our findings suggest that the senescence-associated proliferative arrest seen in genomically unperturbed cultured human epithelial and fibroblast cells may be primarily a consequence of exposure to telomere length-independent stresses rather than telomere attrition.

Materials and Methods

Cell culture. Finite lifespan pre-stasis HMEC from specimens 184 (batch D), 48R (batch T), and 240L (batch B) and post-selection HMEC 184 (batch B; agonescence at passage ~15), 48R (batch S; agonescence at passage ~23), and 240L (agonescence at passage ~18) were obtained from reduction mammaplasty tissue of women ages 21, 16, and 19 years, respectively. Cells were initiated as organoids in primary culture in either serum-free MCDB170 medium (Lonza) plus supplements (2) or serum-containing medium MM (1) or M85 and subjected to multiple partial trypsinizations as described (18). Post-selection HMEC were cultured in MCDB170 as described (2, 18, 19). M85 medium is composed of 50% MM medium and 50% bicarbonate-free supplemented MCDB170; M87 medium is composed of 50% MM4 (MM without the conditioned medium; ref. 1) and 50% supplemented MCDB170. Cholera toxin was added to M85 and M87 at a final concentration of 0.5 ng/mL and oxytocin (Bachem) at 0.1 nmol/L. M85A and M87A media were supplemented with 0.1% AlbuMAX I (Invitrogen). Fibroblasts from specimens 184, 48, and 240L were obtained by growing primary reduction mammaplasty cells in DMEM/F12 with 10% fetal bovine serum and 10 µg/mL insulin; 250 MK cells were obtained from aspirated milk fluids.³

Total population doubling level for each culture was calculated beginning at passage 2 using the formula: population doubling = $\log_2(N_{\text{final}}/N_{\text{initial}})$, where N_{initial} is the number of cells seeded in a dish at each passage and N_{final} is the number of cells recovered from the dish. No corrections were made for plating efficiency.

Immunohistochemistry and immunofluorescence. Immunohistochemical analysis for p16 was done as described using the JC8 antibody (20). SA-β-Gal activity was determined as described (21). Immunofluorescence analysis was done on cells plated on 4- or 8-well chamber slides (Lab-Tek) as described (9) using 10% goat serum in CAS-block (Zymed) as blocking agent for DNA damage markers and 5% goat serum for cytokeratins and cell surface markers. For DNA damage assays, cells were either irradiated with 10 Gy ionizing radiation or mock-irradiated. Antibodies used for immunostaining are listed in Supplementary Table S1. Stained cells were imaged with a Zeiss Axiovert 200 M inverted fluorescence microscope and with a Retiga EX camera (Q-Imaging) and Image-Pro Plus software (Media Cybernetics). Labeling index was determined using immunohistochemistry by labeling cells in 35 mm dishes with 10 µmol/L bromodeoxyuridine for 24 h. Labeled cells were fixed with 70% ethanol, rehydrated, and treated with 0.1 N HCl/0.08% pepsin to denature DNA. Cells were incubated with anti-bromodeoxyuridine antibody (Sigma) overnight at 4°C and bound antibody was detected using the peroxidase mouse ABC kit and DAB substrate kit (Vector Labs).

Mean telomere restriction fragment length and reverse transcription-PCR. Mean telomere restriction fragment (TRF) analysis was done using the TeloTAGGG chemiluminescent telomere length assay (Roche) following the manufacturer's protocol. Genomic DNA was isolated using the Wizard genomic DNA isolation kit (Promega) or by column purification (Qiagen) and 3 µg were digested and resolved on 0.8% agarose gels. The separated DNA was transferred to a membrane, hybridized overnight to a digoxigenin-labeled telomere-specific probe, and washed to remove nonspecific hybrids. Chemiluminescent signal was detected with Kodak Biomax film and quantitated using ImageQuant software (Molecular Dynamics). Mean TRF length was calculated as described (22). Quantitative reverse transcription-PCR was done using the Roche LightCycler and FastStart DNA Master SYBR Green I reagents. First-strand cDNA synthesis used the SuperScript III synthesis system for reverse transcription-PCR (Invitrogen). Transcript levels were normalized to expression of H6PD.

Fluorescence-activated cell sorting. Cells were fed 48 and 24 h before harvest, collected by trypsinization, rinsed with PBS, and fixed with 70% ethanol. Fixed cells were rehydrated in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20, treated with RNase A (1 µg/mL), and stained with propidium iodide (50 µg/mL). Analyses of total DNA content were done using a Becton Dickinson FACScan flow cytometer. The fraction of cells in each cell cycle phase was calculated using the Mod-Fit software program (Verity Software House).

Karyology. HMEC grown in M85 ± oxytocin were exposed overnight to a 0.01 µg/mL concentration of colcemid (Life Technologies) when they were around one passage from stasis. Following trypsinization, metaphase cells were collected in hypotonic buffer (0.075 mol/L KCl) and incubated at 37°C for 30 min followed by fixation in 3:1 methanol/glacial acetic acid. Trypsin G-banding was done following standard procedures (23).

RNA isolation and GeneChip hybridization. Subconfluent cultures were harvested for RNA 24 h following feeding. Cells were lysed with Trizol reagent (Invitrogen) and RNA was isolated according to the manufacturer's protocol. RNA was further purified by column chromatography (Qiagen) and RNA quality was verified using an Agilent 2100 BioAnalyzer. RNA samples for gene expression analysis were processed and hybridized to Affymetrix HT HG-U133A chips containing ~22,000 probe sets with ~13,000 well-annotated genes (Affymetrix) at the Lawrence Berkeley National Laboratory Gene Expression Core Facility.

Statistical and bioinformatics analyses. The raw probe-level intensity data captured in .cel files for each chip were processed for background correction, normalization, and summarization using Probe-Level Linear Models in Linear Models for Microarray data module in Bioconductor package⁴ (24). Quality measures such as the 3'/5' ratios of housekeeping genes, RNA degradation indices, and Normalized Unscaled Standard Error

³ Details on the derivation and culture of these HMEC can be found on our Web site (<http://hmec.lbl.gov>).

⁴ <http://www.bioconductor.org/packages/bioc/1.6/src/contrib/html/affyPLM.html>

values⁵ were used to assess the data, leading to four outlier chips in the data set being excluded from downstream analysis. Probe sets with intensities 2σ below average background across the chips were filtered out, leaving a total of 9,702 genes (13,791 probe sets). Differentially expressed genes were identified by the empirical Bayes linear model fit in Linear Models for Microarray data (25). *P*-value adjustment for multiple testing was done to control the false discovery rate (26). Genes were considered differentially expressed with the following criteria: (a) ≥2-fold differential (positive/negative) expression between the means of two comparison groups and (b) false discovery rate <10%. Volcano plots were generated using Spotfire version 199.1008.⁶

Cluster analyses were done using the Cluster 3.0 program and visualized using Java TreeView. Normalized log₂ gene expression values for stasis and agonoscent HMEC were centered by subtracting the mean value of each probe set across the indicated HMEC samples from each measured value. Unsupervised clustering was done using the 603 genes where one or more samples had a log₂ expression difference of at least ±1.5. Supervised clustering was done on the 77 genes with the highest variance across all HMEC samples; these were clustered gene-wise with correlation coefficient as a distance measure using the average linkage method (27). Principal Component Analysis (28) was done using Partek Genomics Suite (version 6.3, © 2008; Partek). Gene Ontology functional classification of differentially expressed genes from each groups were done using Database for Annotation, Visualization, and Integrated Discovery (29).

Results

Increased proliferative potential of pre-stasis HMEC. Candidate medium formulations were tested using frozen stocks of second passage reduction mammaplasty-derived 184 HMEC that had been grown as primary cultures using MM medium. Primary cultures were initiated using intact organoids, which we have shown provide greater population doubling potential than dissociated single cells (18, 30). These preliminary studies indicated that a medium formulation, M85 [consisting of a 1:1 ratio of MM + cholera toxin (1) and serum-free MCDB170 + supplements (2)], increased population doubling potential, which was further increased by the addition of 0.1 nmol/L of the anti-stress peptide oxytocin. We next initiated primary organoid cultures from three different individuals (reduction mammaplasty specimens 48R, 184, and 240L) in M85 with and without oxytocin (Fig. 1A). Following our early protocols (18, 30), primary cultures were subjected to repeated partial trypsinizations (PTs) to yield multiple second passage cultures that were either stored frozen or maintained in culture. In this protocol, an epithelial monolayer is allowed to grow out from the organoids until subconfluence, ~50% to 70% of the cells are removed by trypsinization, and the culture allowed to regrow to subconfluence and subjected to additional PTs. This protocol precludes precise determination of the number of population doubling in the primary cultures, a value likely to have been at minimum 5 to 10 population doublings and possibly 25 to 30 population doublings after multiple PTs.

Growth in M85 + oxytocin was rapid for 25 to 35 population doublings followed by a gradual slowdown with senescence arrest ~45 population doublings from 2p. In the absence of oxytocin, there was similar initial rapid growth for 20 to 25 population doublings followed by reduced, often heterogeneous, proliferation. Growth from 2p of 184 HMEC in MM is shown for comparison.

We then formulated a medium that could provide long-term growth without the epithelial cell conditioned medium present in MM. Figure 1B show growth from 2p of 184 HMEC in medium containing oxytocin with (M85) or without (M87) conditioned medium in the presence or absence of lipid-rich BSA. In the presence of oxytocin, the BSA was able to substitute for the conditioned medium (M87A + oxytocin curve) and provided more population doublings than the M85 + oxytocin medium. We found no significant differences in long-term growth from 2p of 184 HMEC in M85 + oxytocin and M87 + oxytocin medium under conditions of 20% versus 3% oxygen (data not shown). Our new medium formulations now make possible the generation and frozen storage of large batches of early-passage pre-stasis HMEC that retain extensive proliferative potential, thereby permitting reproducible experimentation using standardized HMEC batches from individual specimen donors.

The M85 + oxytocin medium was used to grow milk-derived HMEC previously grown in MM in primary culture and then stored frozen at 2p (250 MK curve in Fig. 1B). These cells also showed an extended population doubling potential compared with their prior growth in MM (arrest at 10p versus 3p in MM), with a growth curve similar to that seen in our original testing of 184 HMEC started in MM and switched to M85 + oxytocin at 2p (data not shown). The comparison of the 250 MK growth curve with that of 184D HMEC in M85 + oxytocin in Fig. 1B suggests that total population doubling potential is significantly reduced when primary cultures are not initiated in the newer medium formulations.

The pre-stasis cultures of 184D and 48RT HMEC shown in Fig. 1A were examined at different passage levels for markers of growth (labeling index) and senescence (expression of p16 and SA-β-Gal). As shown in Fig. 1C for 184D HMEC, with increasing passage in M85 + oxytocin, the labeling index decreased, whereas the number of cells expressing p16 and SA-β-Gal increased. A few, larger cells expressed senescence markers as early as 2p. As cultures neared stasis at 14p, most cells displayed a senescent morphology (large, flat, and vacuolated) and expressed p16 and SA-β-Gal, although pockets of proliferative cells (small refractile morphology and positive labeling index) were still present. 184D HMEC grown without oxytocin also showed a senescent morphology and expression of p16 and SA-β-Gal, as they approached stasis at 9p. Similar results were seen for 48RT HMEC (data not shown).

We reported previously that, at a first proliferative barrier, 184 and 48R HMEC and isogenic HMFC were arrested in the G₁ phase of the cell cycle (2N to 4N ratio >4) and had normal karyotypes; however, those HMEC cultures had only undergone ~10 population doubling from passage 2 (5). Fluorescence-activated cell sorting examination of near-stasis populations of 184D and 48RT HMEC grown in M85 ± oxytocin also showed a 2N to 4N ratio of >4 (Table 1). Chromosome studies were done on 184D and 48RT HMEC near stasis, yielding 35 analyzable metaphases (Supplementary Table S2). Thirty-four displayed a normal, 46,XX karyotype. One cell showed a recombinant chromosome derived from chromosomes 1 and 3; such single-cell, nonclonal events are frequently observed in cultured normal human cells. These data indicate that although the population doubling level at which stasis occurs depends on culture conditions, the phenotype of HMEC at stasis (senescent morphology, SA-β-Gal staining, low labeling index, expression of p16, arrest in G₁, and normal karyotypes) remains similar and consistent with a retinoblastoma-mediated arrest that is independent of telomere length.

⁵ <http://www.bioconductor.org/docs/vignettes.html>

⁶ <http://spotfire.tibco.com>

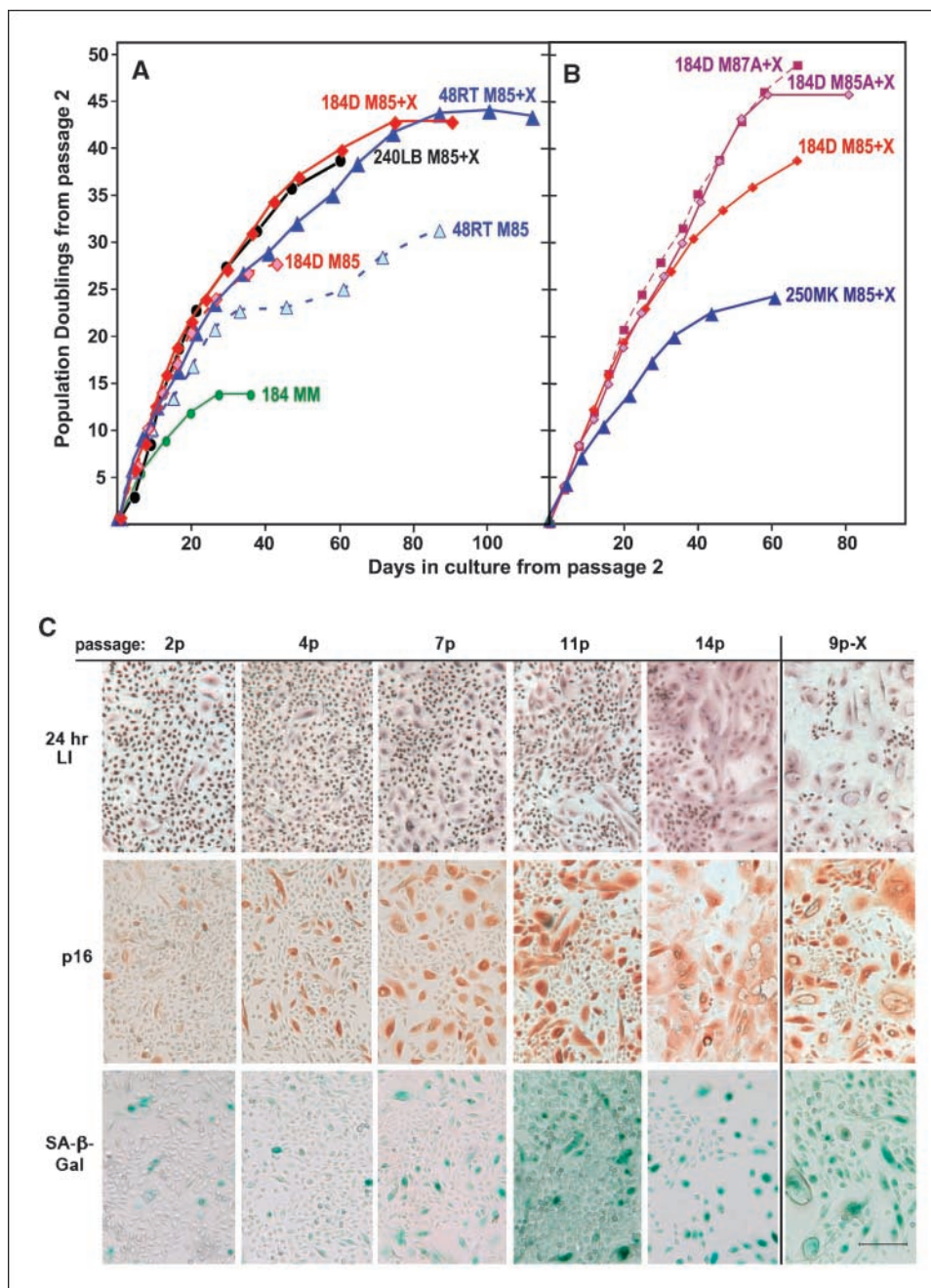


Figure 1. Growth of pre-stasis HMEC in M85 or M87 \pm oxytocin or BSA. **A**, primary cultures from three reduction mamoplasty specimens were started from organoids and grown in M85 \pm oxytocin (X). The number of population doubling in primary culture cannot be accurately determined; growth is shown starting from 2p with cells obtained from PT2. Each point represents cell counts at successive passage levels, for example, the final points for 184D, 48RT, and 240LB grown in M85 + oxytocin are 16p, 17p, and 11p, respectively. All media contained cholera toxin from passage 2. **B**, frozen stocks of second passage 184D HMEC from PT3 were grown in medium with (M85) or without (M87) conditioned medium \pm lipid-rich BSA (**A**) in the presence of oxytocin. 250 MK are derived from milk, grown in MM for primary culture, and then switched to M85 + oxytocin at second passage. **C**, expression of markers associated with proliferation [labeling index (LI)] and senescence (p16, SA- β -Gal) in pre-stasis 184 HMEC with increasing passage. All cells, except for 9p-X, were grown in M85 with oxytocin; stasis in this population was at 15p. The 9p-X culture was grown in M85 without oxytocin; stasis was at 10p. Cultures examined are from the growth curve shown in **A**. Note the reciprocal relationship between the small cells with a positive labeling index and the larger, often vacuolated cells that are positive for p16 and SA- β -Gal and negative for labeling index. Size marker = 200 μ m.

Stasis is independent of mean TRF length. Our previous studies reported that post-selection HMEC did not have detectable telomerase activity and showed ongoing telomere erosion, with evidence of telomere dysfunction when the mean TRF declined to ~ 4 to 5 kb (5, 9). In contrast, low telomerase activity was seen in some pre-stasis HMEC (15, 31), and mean TRF values at stasis were not critically short (~ 8 and ~ 6 kb when grown in MM or MCDB170, respectively; refs. 5, 31). However, the 184 and 48R HMEC examined in those studies grew for only ~ 15 to 30 total population doublings. We now determined whether the extended proliferative potential of pre-stasis HMEC in M85 \pm oxytocin led to critically short mean TRF lengths at stasis.

184D HMEC examined at increasing passages (Fig. 2) showed very gradual TRF attrition between passages 2 and 11. Mean TRF

length in 184D grown with oxytocin declined from ~ 10 to 11 kb at 2p to ~ 9 kb at 11p followed by a steeper decline toward stasis, with a mean TRF length of ~ 7 kb at 14p (~ 45 –55 total population doublings). Without oxytocin, the 184D cultures reached stasis at passage 10 and had a mean TRF of ~ 9.5 kb at 9p (~ 30 –40 total population doublings), similar to the value seen at the same population doubling level in the cultures with oxytocin. These results indicate that the mean TRF value at stasis varies depending on the culture conditions and is not critically short even after >40 population doublings, consistent with the observed normal karyotype at stasis.

Transcript and protein expression in pre-stasis HMEC. Pre-stasis HMEC populations were characterized for gene transcript profiles and protein expression, with emphasis on examining

Table 1. Fluorescence-activated cell sorting analysis of growing and senescent HMEC

Cell ID	Medium	Passage	Status	G ₀ -G ₁	S	G ₂ -M
184D	M85 + oxytocin	4	Growing	60.9*	20.2	18.9
184D	M85 + oxytocin	15	Stasis	89.4	0	10.6
184D	M85	10	Stasis	81.8	9.7	8.4
184B	MCDB170	10	Growing	62.4	19.2	18.4
48RT	M85 + oxytocin	14	Stasis	87	0.4	12.6
48RT	M85	10	Stasis	88	3.0	9.0

*Percentage of cells in the different cell cycle phases.

markers of mammary cell lineage and the effects of passage, senescence, and interindividual differences. 184D and 48RT HMEC were examined for lineage markers by immunofluorescence at 5p and 13p as well as 250 MK at 3p; additional cultures were examined at various passages by immunohistochemistry for K19. The 184D, 48RT, and 240LB HMEC grown in M85 ± oxytocin shown in Fig. 1A were examined for gene transcript profiles with increasing passage levels; growing 250 MK HMEC were also analyzed (Fig. 3A). Additionally, growing (184B, 48RS, and 240L) and agonescent (184B and 48RS) post-selection HMEC and growing and senescent 184 and 48 HMFC were examined to permit comparisons with the pre-stasis cells (see below). A more extensive analysis of the gene transcript data will be the subject of a separate publication.

Figure 3B to D shows cellular morphology and the expression of lineage-specific proteins by light microscopy, immunohistochemistry, and immunofluorescence. Figure 3B illustrates the heterogeneous morphology of 184D passage 3 cultures derived from different PTs. Cells from PT2 contain mostly cobblestone epithelial cells and flatter cells in closed colonies similar to what has previously been described as a luminal morphology (32); cultures

from PT16 additionally display a cell type with thin cytoplasmic extensions across other HMEC. As expected, HMEC in the closed colonies stained positive for the luminal marker K19, as did some cells with a more cobblestone appearance (cultures shown from PT15). Figure 3C illustrates protein expression in 48RT passage 5 cultures of genes associated with specific lineages *in vivo*: muc1 and EpCam are expressed by luminal lineages, K14 by myoepithelial lineages, and K19 by both luminal and stem lineages (33). K19-positive, EpCam-positive, and muc1-positive cells were seen either interspersed among cells with myoepithelial markers or as small homogenous colonies. Figure 3D illustrates the expression of luminal markers by almost all the milk-derived 250 MK cells at passage 3, indicating that our new medium formulations are capable of propagating homogenous luminal HMEC populations.

Figure 3A shows a hierarchical cluster of the gene levels of 77 genes chosen from the top 200 genes with greatest variance across all epithelial samples plus several handpicked lineage or differentiation-associated genes (*EGFR*, *KRT14*, *KRT18*, *KRT7*, *PGRMC2*, *RARA*, and *RARB*). Pre-stasis HMEC specimens from the Fig. 1A curves ±

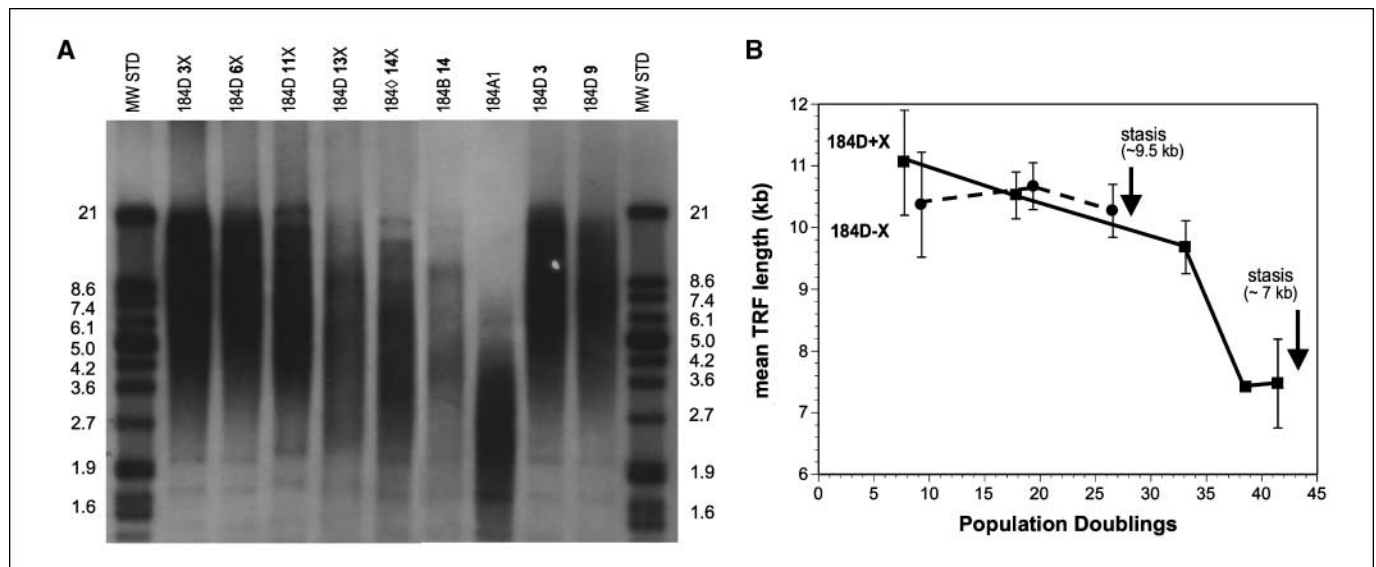


Figure 2. Mean TRF length of pre-stasis 184D HMEC with increasing passage. Pre-stasis HMEC are from Fig. 1A. Agonescent post-selection HMEC (184B 14p) and the immortalized line 184A1 are shown for comparison; 184A1 has a reported mean TRF length of ~3 to 5 kb and agonescent 184 HMEC have a reported faint signal with a mean TRF of ≤5 kb (15, 50). Molecular weight standards (MW STD) are shown on the outside lanes. A, representative gel. B, calculated mean TRF values from three independent gels (one sample only at 13p).

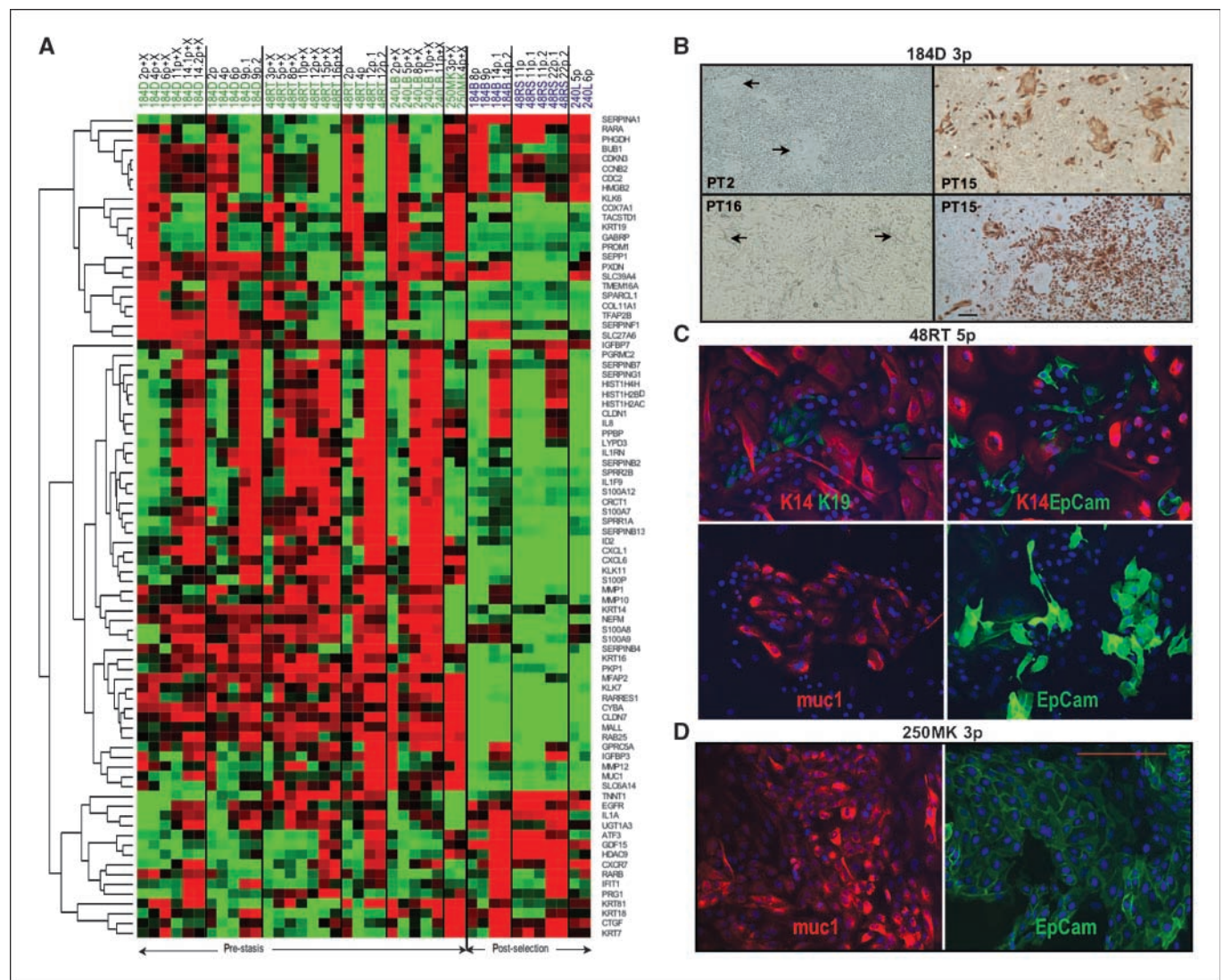


Figure 3. Gene transcript and protein expression in pre-stasis HMEC from different individuals. **A**, hierarchical clustering (by rows) of gene transcript profiles in growing and senescent pre-stasis and post-selection HMEC. Pre-stasis 184D, 48RT, and 240LB HMEC (from Fig. 1A) are shown in columns with increasing passage up to stasis. X, growth in oxytocin. Post-selection 184B and 48RS HMEC are growing and agonescent populations. Genes shown are 77 selected for the greatest variance across all samples plus a few selected lineage or differentiation-associated genes. **B**, morphology and K19 expression in pre-stasis 184D HMEC grown in M85 + oxytocin at passage 3: a culture from PT2 shows cobblestone and closed colony (arrows) morphologies, whereas a culture from PT16 shows more heterogeneous morphologies, including cells with cytoplasm that extends over other cells (arrows). Cultures from PT15 stained for K19 by immunohistochemistry show the presence of K19 protein in cells with the closed colony morphology as well as in some areas with cobblestone and extended morphologies. Size marker = 200 μ m. **C**, lineage markers in 48RT passage 5 (from PT5) stained by immunofluorescence. Cultures show cells displaying luminal markers (K19, EpCam, and muc1) interspersed with cells displaying myoepithelial lineage marker (K14) and as small homogeneous patches. **D**, lineage markers in 250 MK passage 3 illustrate a luminal phenotype. Size marker = 200 μ m.

oxytocin are arranged by increasing passage level. The 250 MK samples represent cells with a luminal phenotype, whereas the post-selection HMEC are known to express mostly myoepithelial lineage markers (32).

The data in Fig. 3 illustrate several points about the pre-stasis HMEC. First, genes and proteins of both myoepithelial and luminal epithelial cell lineages are expressed in the pre-stasis populations derived from reduction mammaplasties; however, unlike the milk-derived 250 MK, cells with luminal markers made up only 10% to 25% of the populations. The immunofluorescence and immunohistochemistry results (Fig. 3B-D) are corroborated by the gene transcript data (Fig. 3A), showing expression of K19, EpCam (*TACSTD1*), and muc1 in all pre-stasis specimens. Thus, our new

medium can support long-term growth of reduction mammaplasty-derived cells with multiple lineage markers. Second, expression levels of many genes change as the cultures progress from early passages to stasis, with most of the changes seen as the populations approach stasis. This result is not surprising, given the differences in proliferation rate as well as known differences in gene expression at senescence in many cell types. However, it also points out the importance of population doubling level as a significant variable to be identified when examining or comparing normal pre-stasis HMEC. Third, although the transcript profiles from the three individual reduction mammaplasty donors are largely similar, some interindividual differences are present (see also Supplementary Figs. S1 and S2), highlighting the importance of examining

multiple individuals. Finally, we observed that the morphology of the cells growing out from the primary organoid cultures changed with ongoing partial trypsinizations (Fig. 3B). Current studies are using pre-stasis HMEC sorted by lineage markers to identify lineage-specific gene expression.

Altogether, these studies show that pre-stasis HMEC grown in our new formulations provide well-characterized normal populations that maintain long-term active growth of multiple mammary cell types similar to what is seen *in vivo*. All these HMEC ceased proliferation with a phenotype characteristic of stasis; no spontaneous escape from stasis of any cell growing in any of these serum-containing medium has been observed. These long-term pre-stasis cultures were then used, as described below, to compare growing and senescent populations of pre-stasis and post-selection HMEC and isogenic HMFC to further define the molecular properties of pre-stasis versus post-selection HMEC and the stasis versus telomere dysfunction proliferation barriers.

DNA damage markers in growing and senescent pre-stasis and post-selection HMEC and isogenic HMFC. Cultured human fibroblasts nearing senescence have been reported to show increased expression of 53BP1 and γ H2AX foci and Ser¹⁵ phosphorylated p53, markers usually attributed to DNA damage (34, 35). Our previous data and model of the senescence barriers (9) predicted that significant DNA damage would be present at agonescence, but we could not predict the situation for HMEC at stasis. We now used immunofluorescence to examine and compare the level of 53BP1 and γ H2AX foci and Ser¹⁵ phosphorylated p53 in growing and senescent pre-stasis and post-selection HMEC and isogenic HMFC to determine if HMEC stasis was associated with markers of DNA damage.

HMEC and HMFC were examined while actively growing or within one passage from proliferative arrest. 53BP1 foci data are shown in Fig. 4A and Supplementary Table S3, and representative immunofluorescence images for 53BP1 and γ H2AX foci are shown in Fig. 4B. Representative images for Ser¹⁵ phosphorylated p53 are shown in Fig. 4C and D. Exposure to ionizing radiation was a positive control. Most of the HMEC populations at stasis and agonescence, as well as the senescent fibroblasts, have ≥ 1 53BP1 focus per cell. However, the HMEC at stasis, and the senescent fibroblasts, have significantly fewer cells with ≥ 3 foci compared with the HMEC at agonescence, which were more similar to the irradiated cultures in the number of foci and expression of activated p53. More foci were detectable in the growing post-selection versus pre-stasis HMEC and there was a higher number of foci in all 184-derived populations compared with 48R. Notably, the number of foci in the pre-stasis cultures increased with passage, rather than correlating with stasis per se, as the cultures maintained without oxytocin reached stasis 4 to 5 passages (~ 15 -20 population doublings) earlier, with fewer foci than the cultures grown for more population doublings with oxytocin. Similar to the data for 53BP1 and γ H2AX foci, activated p53 was evident in both 184B and 48RS HMEC at agonescence and was lower (184D) or barely detectable (48RT) at stasis. Surprisingly, we did not detect activated p53 in either 184 or 48 HMFC at senescence.

These data are consistent with the model that agonescence is a consequence of DNA damage signals resulting from telomere attrition, whereas the longer mean TRF and absence of genomic instability in the cultures at stasis argue that the lower level of foci seen at stasis are not the result of telomere dysfunction due to telomere attrition. Ongoing proliferation may create conditions that elicit low levels of DNA damage independent of telomere

attrition (34). Diversity among donors in vulnerability to DNA damaging stresses might also influence the level of DNA damage foci observed, possibly accounting for the observed differences between cells from specimens 184 and 48R. Altogether, these results indicate that HMEC stasis is not directly correlated with levels of DNA damage.

Comparison of gene expression in growing and senescent pre-stasis and post-selection HMEC and isogenic HMFC. To further define the molecular differences and similarities between HMEC arrested at stasis versus agonescence, the transcriptional profiles of growing and senescent populations of pre-stasis and post-selection HMEC were compared. Additionally, isogenic senescent HMFC were compared with the HMEC at stasis and agonescence.

Principal Component Analysis was applied to visualize correlations in the HMEC and HMFC transcriptional profiles. Figure 5A illustrates that HMEC populations can be distinguished using the first three principal components, which account for 37% of total variation. Several observations can be made. (a) Growing populations were separated from senescent populations along the first principal component. Pre-stasis cultures with mixed growing and senescent cells showed intermediate positions. (b) Pre-stasis cultures were separated from post-selection cultures along the second principal component; cells at stasis are separated from cells at agonescence, and growing pre-stasis and post-selection cells are separated. The distinctions in the transcriptional profiles of HMEC at stasis and agonescence, as well as the similarities, are further illustrated by hierarchical cluster analysis (Supplementary Fig. S1), which also shows some interindividual differences. Figure 5B shows the Principal Component Analysis comparing senescent HMFC with HMEC at stasis and at agonescence; three principal components accounted for 54% of the total variation. Senescent HMFC are strikingly separated from both types of senescent HMEC along the first principal component, whereas the HMEC populations at stasis and agonescence were distinguished along the second principal component. These results indicate a strong cell type-specific contribution to senescent gene expression profiles. Figure 5C compares genes modulated in senescent pre-stasis and post-selection HMEC and HMFC in relation to their growing populations. A large number of genes were unique to either stasis (451) or agonescence (577), although many genes were modulated at both stasis and agonescence (370). Very few genes that were modulated in senescent HMFC were also modulated in HMEC at stasis or agonescence, and only two genes were in common in all the senescent populations. Figure 5D represents the fold changes and statistical significance of genes differentially modulated in expression between stasis and agonescence. Functional analyses showed that genes associated with "response to stress" were up-regulated at both barriers, but there was no overlap among the gene in the lists (data not shown).

Ten genes with varying expression among the growing and senescent HMEC populations were chosen for quantitative reverse transcription-PCR verification of expression levels (Supplementary Fig. S2) using the primers shown in Supplementary Table S4; all results were consistent with the microarray data, although levels of expression varied between HMEC derived from specimens 184 and 48R.

Altogether, these data indicate that the HMEC senescence barriers of stasis and agonescence are associated with distinct transcriptional profiles, with some similarities in their gene

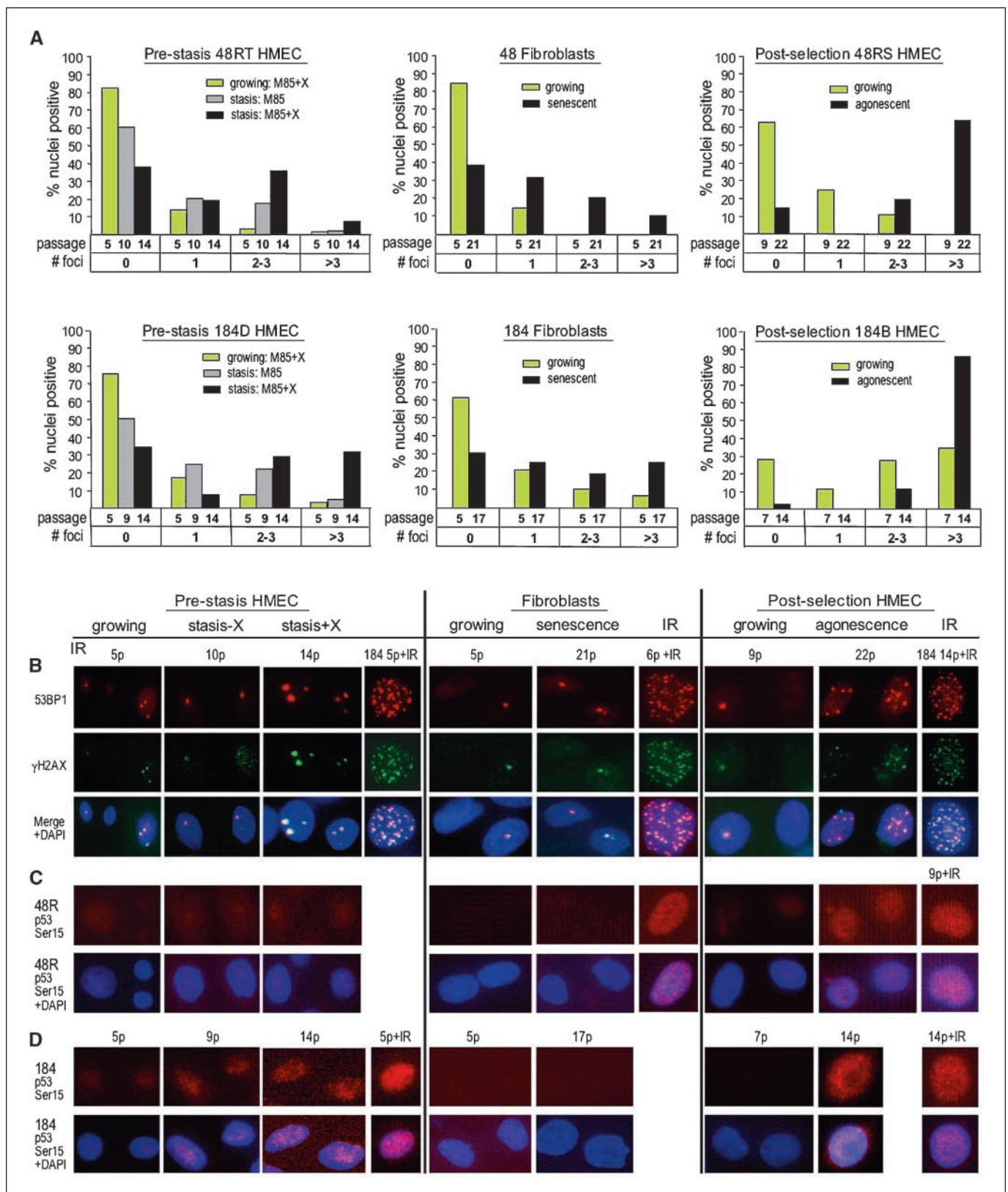


Figure 4. Markers of DNA damage in growing and senescent pre-stasis and post-selection HMEC and isogenic HMFC. Senescent cultures were examined one passage before when cultures showed no net increase in cell number. **A**, 53BP1 foci. Note the higher level of foci in agonescent HMEC compared with senescent HMFC or HMEC at stasis and the higher level of foci in cells from specimen 184 compared with specimen 48. **B** to **D**, representative immunofluorescence images of growing and senescent pre-stasis and post-selection HMEC and isogenic HMFC. **B**, 53BP1 and γ H2AX foci in specimen 48. **C** and **D**, activated p53 in specimens 48 and 184. Cells exposed to 10 Gy ionizing radiation (*IR*) are shown as positive controls. Note the greater number of foci and higher level of Ser¹⁵ phosphorylated p53 in agonescent HMEC compared with senescent HMFC or HMEC at stasis. Cultures examined are from the growth curve shown in Fig. 1A.

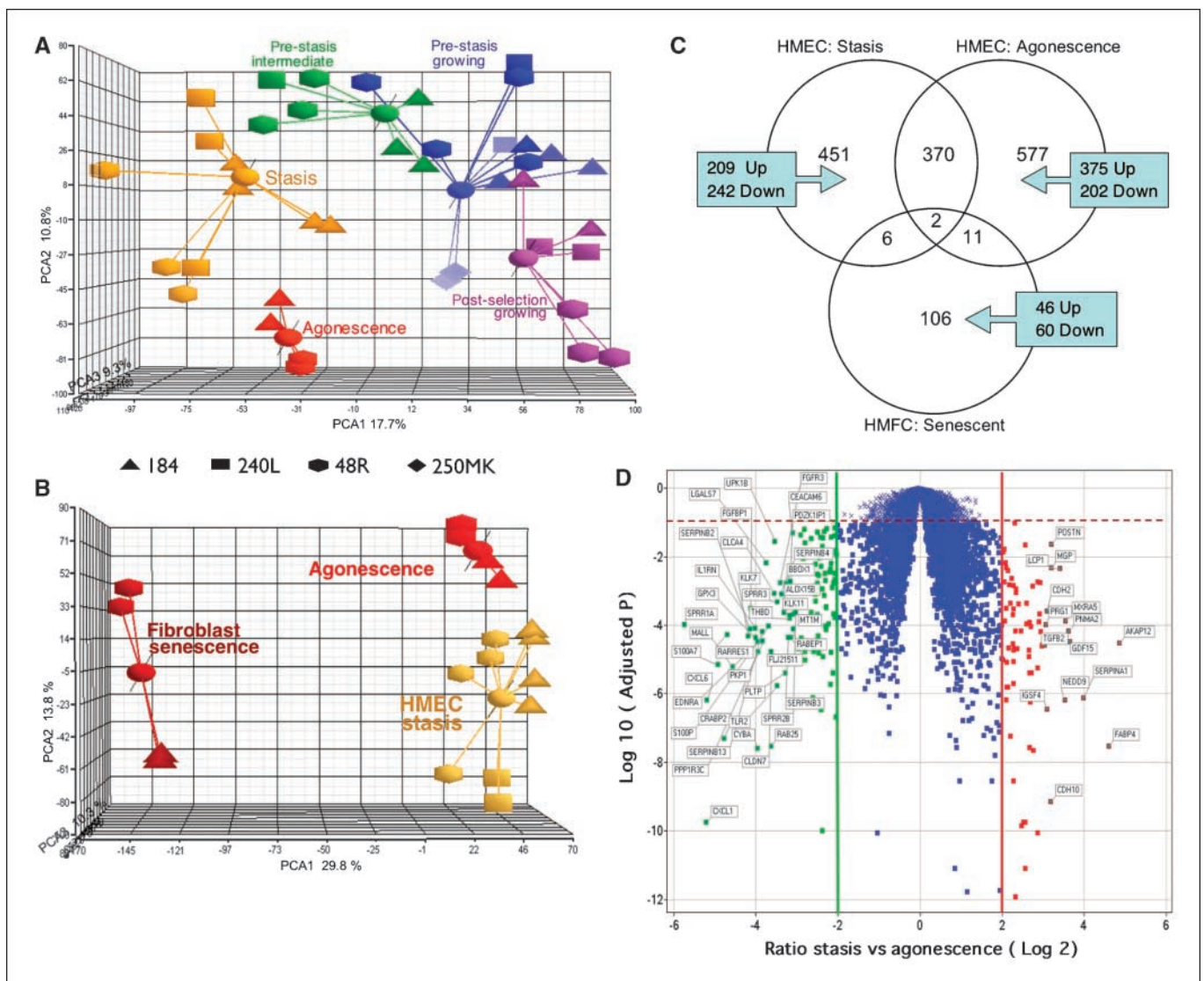


Figure 5. Relationship of growing and senescent HMEC and HMFC as determined by transcriptional profiles. *A*, Principal Component Analysis plot of transcriptional profiles of growing and senescent pre-stasis and post-selection HMEC. *B*, Principal Component Analysis plot of transcriptional profiles of all senescent HMEC and HMFC. The three-dimensional scatter plots show the first three principal components of the analysis of 9702 genes. Data points from different individuals are represented by different shapes. *C*, Venn diagram of genes modulated at HMEC stasis using growing pre-stasis as baseline, at HMEC agonescence using growing post-selection as baseline, or at HMFC senescence with growing fibroblast as baseline. Diagram depicts the number of genes unique to each group and the number that overlap between and among the groups. *D*, volcano plot illustrating genes significantly differentially expressed between stasis versus agonescence. X axis, fold change ratio (\log_2) between HMEC at stasis and agonescence; Y axis, significance with adjusted P values. The graph is segmented to represent the genes that satisfy the fold change criteria of ± 2 and adjusted $P < 0.1$. Segments on the right and left show the genes up-regulated at agonescence (79) and stasis (116), respectively. Genes with >8 -fold change difference between stasis and agonescence are labeled.

expression. In contrast, the transcriptional profile of genes modulated in senescent HMFC showed very little overlap with the profiles seen in either type of senescent HMEC.

Discussion

Malignant transformation of normal human epithelial cells requires overcoming tumor suppressor barriers that induce cellular senescence in response to stresses, DNA damage, and telomere attrition. Understanding the molecular bases of these senescence barriers, and how cells bypass or overcome them, can provide valuable information relevant to the etiology of and therapeutic intervention in malignant progression. Based on our long-term

studies, we have previously proposed a molecularly defined model of the senescence barriers encountered by cultured HMEC (ref. 9; Supplementary Fig. S3) that is consistent with observed carcinogenic progression *in vivo*. Three distinct types of senescence barriers have been observed (5, 9, 36): stasis, a stress-associated barrier mediated by retinoblastoma that arrests cells in G₁ with a low labeling index and normal karyotypes; telomere dysfunction due to telomere attrition that produces genomic instability; and oncogene-induced senescence that involves a DNA damage response. Molecular definition of human cell senescence barriers has been hampered by variability among species and cell types in vulnerability and responses to senescence-inducing conditions. Additionally, the limited growth potential of cultured normal

human epithelial cells before the onset of stasis has constrained experimental examination of these cells. In this report, we describe simple culture methods that support long-term active growth of normal pre-stasis HMEC that express markers of multiple mammary cell lineages and the use of these cultures to delineate molecular distinctions between the stasis and telomere dysfunction senescence barriers. These data lend further support to our proposal that the senescence barrier encountered by genomically unperturbed human epithelial and fibroblast cells in culture may be primarily a consequence of exposure to telomere length-independent stresses rather than telomere attrition (9).

Our model of the senescence barriers encountered by cultured HMEC proposed that the observed variable population doubling potential of HMEC before a first proliferative arrest is due to variable stress exposure under different culture conditions (9). Here, we show that simple changes in medium composition greatly extend the population doubling potential of HMEC before a p16-positive stasis arrest. We used medium formulations that combined components of our previous HMEC medium, serum-free MCDB170 (2), with serum-containing and conditioned medium-containing MM (that is, M85), or with MM4, which lacks the conditioned medium (that is, M87; refs. 1, 30), plus the anti-stress peptide oxytocin and/or lipid-rich BSA to achieve ~60 population doublings from reduction mammaplasty-derived HMEC before stasis. Proliferation was rapid (~50 population doublings in 65 days from passage 2) and did not require any special treatment of tissue culture substrates, in contrast to a recent report using the treated Primaria dishes and WIT medium, which achieved ~42 population doublings total over ~130 days from passage 1 (37). The addition of oxytocin alone gave ~20 population doublings of increased proliferation, although it had little, if any, effect on the rapid initial growth rate, suggesting that it acts mainly to delay stasis rather than stimulate proliferation. Although the role of oxytocin in reducing stress responses at the whole body level is well known (38), its use to prolong normal human cell growth in culture has not, to our knowledge, been previously reported. Oxytocin signals via G-coupled receptors, which are found on breast cancer cells, and uses the phosphoinositide 3-kinase and mitogen-activated protein kinase signal transduction pathways (39–42). Oxytocin has been shown to inhibit proliferation of some human tumor-derived cell lines, including breast, associated with increasing cyclic AMP levels, while stimulating other tumor lines (40) and primary explant cultures of rodent mammary myoepithelial cells (43). Further studies will be needed to elucidate how oxytocin delays the onset of stasis in normal HMEC.

Our new medium formulations that support long-term growth of pre-stasis HMEC should facilitate experimental examination of normal HMEC biology. This is particularly important as many studies of “normal” HMEC currently use the p16-negative post-selection HMEC, which have been shown in this report and others to display properties distinct from normal pre-stasis HMEC in culture and *in vivo* (9–14). Notably, as shown by immunofluorescence, immunohistochemistry, and gene transcript profiles, our culture conditions supported long-term growth of HMEC with both luminal and myoepithelial lineage markers. Luminal epithelial markers were seen on ~10% to 25% of reduction mammaplasty-derived cells, with decreased expression of the luminal markers K19 and EpCam with increasing passage. This heterogeneity contrasts with the post-selection HMEC populations, which display predominantly myoepithelial markers at all passages, as well as some limited luminal epithelial marker expression (e.g., sialomu-

cins) but no K19 (7, 32). Growing milk-derived HMEC displayed a luminal phenotype. Quantitative analysis of the different lineages present in pre-stasis HMEC by fluorescence-activated cell sorting using lineage markers is currently in progress. Interindividual differences were noted in gene expression patterns, particularly at stasis, as well as in levels of DNA damage markers. Together with some of our previous studies showing interindividual differences (44), these results point to the importance of examining HMEC from more than one individual when assessing normal HMEC properties. Our findings also illustrate how markers related to growth and stasis, such as p16, SA- β -Gal, labeling index, and gene expression, varied continuously with passage level, such that information about population doubling level examined relative to total population doubling potential of that population can be of crucial significance for interpretation of results or comparison among cell populations.

Pre-stasis populations also differed from post-selection HMEC in parameters related to telomere dynamics, genomic stability, and DNA damage. Previous studies showed that agonescence correlates with critically short telomeres (mean TRF ≤ 5 Kb), a p53-dependent DNA damage response, and widespread genomic instability (5, 9). Virtually all cell metaphases at agonescence showed gross chromosomal abnormalities and telomere associations. This result indicates that the p53-mediated growth arrest at HMEC agonescence does not occur as soon as a single uncapped telomere appears. Rather, HMEC senescence resulting from telomere attrition is associated with critically short telomeres and genomic instability. In contrast, pre-stasis cultures even after >50 population doublings reached stasis with a normal diploid chromosome complement, thus avoiding the acquisition of many uncharacterized genomic errors as occurs in post-selection HMEC approaching agonescence. Determination of mean TRF lengths indicated, as expected from previous studies, that stasis occurred independent of telomere length. In the situations we have observed thus far, stasis occurred before the appearance of critically short telomeres, with a mean TRF of ~6 to 9 kb. However, it is theoretically possible that normal pre-stasis HMEC could proliferate to the point of reaching critically short telomeres and agonescence before encountering stasis if grown under very low stress culture conditions. Consistent with the noncritically short TRF length and genomic stability of HMEC at stasis, cells at stasis exhibited greatly reduced levels of markers of DNA damage (53BP1 and γ H2AX foci, Ser¹⁵ phosphorylated p53) compared with cells at agonescence. Levels of DNA damage markers increased with increasing population doubling level and did not correlate with stasis per se, arguing against HMEC stasis being a consequence solely of DNA damage signals.

Altogether, the comparisons shown here and previously of growing and senescent pre-stasis and post-selection HMEC support our model (Supplementary Fig. S3) of two molecularly distinct senescence barriers: stasis, resulting from chronic or acute stresses, and telomere dysfunction (agonescence or crisis) due to telomere attrition. Our model is consistent with observations from *in vivo* carcinoma progression; early-stage lesions frequently exhibit errors in the retinoblastoma pathway (e.g., loss of p16 expression), whereas ductal carcinoma *in situ* is associated with short telomeres and genomic instability (16). *In vivo*, HMEC may also be subject to oncogene-induced senescence, and errors that allow this barrier to be overcome could influence the ability of the cells to overcome stasis and/or telomere dysfunction.

Transcriptional profiles indicated substantial differences between HMEC stasis and agonescence as well as between both types of HMEC senescence and HMFC senescence. Bioinformatics analyses showed that the majority of genes modulated at stasis and agonescence were distinct, although there was also significant overlap. The transition from growing post-selection to agonescence was associated with inflammation and telomere processes, which have been implicated in cancer progression. Bioinformatics analyses also showed that HMFC use different biological processes than HMEC when transitioning from growing to senescence. As HMFC senesced, they up-regulated genes associated with cell communication and down-regulated genes associated with cell differentiation. The differential utilization of genes between the two transitions in HMEC and the transition in HMFC is remarkable, with only two genes whose modulation is in common between all three transitions. This lack of overlap between genes modulated in HMEC and HMFC senescence is consistent with previous reports comparing post-selection HMEC and mammary fibroblasts, which also showed that genes associated with stress response, cell signaling, cell proliferation, and cell adhesion are significantly enriched in HMEC at what we have called agonescence (45). The striking difference in gene expression between the senescent HMFC and HMEC clearly illustrates cell type specificity in biological processes associated with senescence.

Although the gene transcripts of senescent HMFC were distinct from senescent HMEC, in other properties examined, the senescent HMFC resembled HMEC at stasis and differed from HMEC at agonescence. Like HMEC at stasis, senescent HMFC show a low labeling index, arrest in G₁, expression of p16, absence of critically short telomeres or genomic instability (5), and low or absent DNA damage markers. Given the greater resemblance of senescent HMFC to HMEC at stasis versus agonescence, it is most likely that they too are arresting at a stasis-like senescence barrier rather than as a consequence of telomere dysfunction due to telomere attrition. However, the diversity in senescence parameters among cells from different tissues, organs, and species, exposed to different types of stresses suggests caution in extrapolating results among cell types. We were surprised that activated p53 was not detected in either 184 or 48 HMFC at senescence, although it has been seen in other senescent human fibroblasts (35). This result could be related to prior studies showing elevated levels of p16, but not p21, in these HMFC populations at senescence (5). Both cultured HMEC and HMFC may be similar in their preferential use of p16 and not p21 to enforce stasis. We have speculated that induction of p21 may result from p53-inducing stresses such as endogenous oxidative damage; mammary gland-derived cells may be less vulnerable to oxidative damage than, for example, keratinocytes or fetal fibroblasts.

Oxidative damage may occur preferentially at telomeres due to their high guanine triplet content and less efficient DNA damage repair (46), which can lead to a DNA damage response exhibiting foci at telomeres. Telomere erosion is also accelerated in some cell types exposed to hyperoxia; severe hyperoxia-induced telomere attrition in human fetal fibroblasts led to detectable genomic

instability before a full DNA damage-induced arrest (47). Nonetheless, most reports do not show critically short telomeres or genomic instability in senescent human fibroblasts (48), and their senescence arrest could be, in part, a consequence of an elicited DNA damage response showing preferential damage at the telomeric ends rather than from telomere attrition per se. This distinction is significant because, at least in HMEC, telomere dysfunction from telomere attrition is associated with widespread genomic instability, whereas cells at stasis have normal genomes. Telomerase reactivation is required to overcome the proliferation barrier resulting from telomere attrition but is not necessary or sufficient to overcome a telomere length-independent, p53-dependent DNA damage response. This distinction may also be significant for modeling malignant progression; genomic instability and telomerase reactivation are first seen at the ductal carcinoma *in situ* stage and are not found in atypical hyperplasia (5, 9, 15, 16). We have proposed that the genomic instability seen in HMEC with telomere dysfunction due to telomere attrition may give rise to errors allowing telomerase reactivation (49).

In summary, we have developed culture conditions that allow long-term growth of HMEC with markers found in both myoepithelial and luminal epithelial HMEC lineages *in vivo*. This increased proliferative potential facilitates studies on the molecular properties of growing and senescent normal HMEC as well as their use for high-throughput examination of pro- and anti-aging or carcinogenic factors. The senescence barrier encountered by these cells, stasis, is not associated with critically short telomeres or genomic instability, in contrast to the telomere dysfunction barrier, agonescence, encountered by the p16-positive, p53-positive post-selection HMEC that have overcome stasis. Isogenic HMFC at senescence arrest express properties more similar to HMEC at stasis than agonescence. Our data suggest that the senescence barrier encountered by genomically unperturbed human epithelial and fibroblast cells in culture may be primarily a consequence of exposure to stresses rather than telomere attrition.

Disclosure of Potential Conflicts of Interest

M.R. Stampfer and J.C. Garbe: Patent filed. The other authors disclosed no potential conflicts of interest.

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